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Major review

Systemic and ocular fluid compounds as potential biomarkers in age-related macular degeneration

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ABSTRACT

Biomarkers can help unravel mechanisms of disease and identify new targets for therapy. They can also be useful in clinical practice for monitoring disease progression, evaluation of treatment efficacy, and risk assessment in multifactorial diseases, such as age-related macular degeneration (AMD). AMD is a highly prevalent progressive retinal disorder for which multiple genetic and environmental risk factors have been described, but the exact etiology is not yet fully understood. Many compounds have been evaluated for their association with AMD. We performed an extensive literature review of all compounds measured in serum, plasma, vitreous, aqueous humor, and urine of AMD patients. Over 3600 articles were screened, resulting in more than 100 different compounds analyzed in AMD studies, involved in neovascularization, immunity, lipid metabolism, extracellular matrix, oxidative stress, diet, hormones, and comorbidities (such as kidney disease). For each compound, we provide a short description of its function and discuss the results of the studies in relation to its usefulness as AMD biomarker. In addition, biomarkers identified by hypothesis-free techniques, including

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metabolomics, proteomics, and epigenomics, are covered. In summary, compounds belonging to the oxidative stress pathway, the complement system, and lipid metabolism are the most promising biomarker candidates for AMD. We hope that this comprehensive survey of the literature on systemic and ocular fluid compounds as potential biomarkers in AMD will provide a stepping stone for future research and possible implementation in clinical practice.
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1. Introduction

The term biomarker refers to an objective, measurable characteristic that is indicative of a biological process (normal, pathogenic, or in response to treatment).[30] Biomarkers can help unravel mechanisms of disease and identify (new) targets for treatment. The potential benefit of biomarkers in drug development is to allow earlier, more robust drug safety and efficacy measurements.[385] In addition, biomarkers can be useful in clinical practice for detecting disease, monitoring disease progression, evaluation of treatment efficacy, and risk assessment. Biomarker testing is an important step toward personalized medicine in many diseases, such as cancer,[198] but also in age-related macular degeneration (AMD).

AMD is the leading cause of irreversible loss of vision among the elderly in the Western world, and the prevalence of AMD is expected to increase with population ageing.[364]

The early stage of AMD is characterized by subretinal yellowish deposits, known as drusen, and changes in macular pigmentation.[43,151] At this stage, patients usually express little or no complaints. As AMD progresses, central vision becomes increasingly blurred, resulting in irreversible vision loss in the advanced stages of the disease. Two subtypes of advanced AMD can be distinguished: geographic atrophy (GA) and neovascular AMD (nAMD).[43,151] The atrophic form of AMD is characterized by cell death of the retinal pigment epithelium (RPE) and photoreceptors causing gradual vision loss.[136] Neovascular AMD, also referred to as "wet" or "exudative" AMD, is characterized by abnormal vessel growth into the retina from the choroid (choroidal neovascularization [CNV]). Leakage from these fragile neovascularizations can cause rapid loss of vision.[363] In this review, we will use the following terms for the different AMD subgroups described in literature: any AMD, early AMD, advanced AMD (GA/neovascular/any advanced), and dry AMD (for definitions of these terms, see Table 1).

AMD is a multifactorial disease, and many risk factors for the development of AMD have been described. The most commonly reported environmental risk factors include aging, smoking, family history, low dietary intake of antioxidants and omega-3 fatty acids, and reduced physical activity.[44,192,204] In addition, multiple genetic risk factors have been identified, consisting of genetic variants that are either common or rare in the population. A large risk effect has been reported for genetic variants located at the *CFH* and *ARMS2/HTRA1* loci.[101] Most genes associated with AMD can be clustered into 5 main pathways: the complement pathway, lipid transport, extracellular matrix (ECM) remodeling, angiogenesis, and cell survival.[100] Despite considerable progress in understanding the genetic architecture of AMD, the exact disease etiology is not yet fully understood.

In attempts to unravel the etiology of AMD, to improve patient stratification, to monitor disease progression, and to discover new drug targets, many biomarker studies have been performed. In general, new analytical strategies have emerged, moving from single markers toward complex biomarker signatures, increasing the chance for greater specificity and a higher diagnostic or predictive value.

There has been no comprehensive overview of all potential biomarkers and their applicability in AMD. Here, we present a detailed summary of the current literature on molecular compounds reported as analyzed in serum, plasma, aqueous humor, vitreous, and urine of AMD patients. The scope of this review is limited to nongenetic chemical compounds. For all compounds, a short description of their function is provided, and the results of the studies are summarized and discussed in relation to AMD. Currently, most of these markers are not yet established as routine clinical diagnostic tools and are discussed here to direct future research and eventually clinical implementation.

2. Neovascularization and hemostasis

Because choroidal neovascularization is one of the subtypes of advanced AMD, it is not surprising that the factors involved in neovascularization and hemostasis have been extensively studied. The results of the studies describing these factors are described in Sections 2.1 and 2.2, respectively. A complete overview of the studies and references is provided in Supplementary Table 1.

2.1. *Neovascularization*

2.1.1. *Vascular endothelial growth factor and soluble VEGF receptor 1*

Vascular endothelial growth factor (VEGF) is currently the most important target in the treatment of nAMD, and the expression profile of VEGF has been extensively investigated in AMD patients. VEGF acts as a hypoxia-driven local signal to induce the formation of new blood vessels. Treatments inhibiting its function can partially restore and/or maintain vision in nAMD patients.

Contrary to expectation, VEGF is not consistently upregulated in AMD patients across studies. One study showed that VEGF levels in the aqueous humor of 12 nAMD patients were highly elevated (668.9 pg/mL) compared with 10 controls (cataract patients; 108.3 pg/mL).[334] In a second study involving

Table 1 – Explanation of terms used in this review to describe different types of AMD

Type of AMD	Criteria
Any AMD	No specific definition of AMD reported or analyses were performed on all AMD stages together
Early AMD	Analyses were performed on AMD cases in the absence of advanced stage disease (GA or CNV) and can include early and/or intermediate AMD
Advanced: GA	Geographic atrophy of the RPE secondary to AMD
Advanced: neovascular	Choroidal neovascular lesion (active or occult) secondary to AMD, including serous and/or hemorrhagic RPE detachment, subretinal fibrovascular tissue and scarring
Any advanced AMD	No specific definition of advanced AMD reported or analyses were performed on both advanced AMD stages (GA and CNV) together
Dry AMD	No specific definition of dry AMD reported or analyses were performed on AMD cases in the absence of advanced neovascular AMD (can therefore include early AMD and/or advanced: GA)

AMD, age-related macular degeneration; CNV, choroidal neovascularization; GA, geographic atrophy; RPE, retinal pigment epithelium.

aqueous humor, however, significant higher VEGF levels could only be demonstrated in the most aggressive form of nAMD (type 3 neovascularization) compared with controls.[74] A third study did not report a difference in VEGF levels in aqueous humor between nAMD and controls at all.[290] Of note, a considerable range in VEGF levels in aqueous humor exists among these studies. In the study by Tong and colleagues,[334] the levels of VEGF in control individuals were around 100 pg/mL, whereas the VEGF levels in the 2 other studies were much lower in controls (39.5 pg/mL and 63.9 pg/mL, respectively).[74,290] These differences may be explained by the use of 3 different analytical systems, emphasizing the need for standardized assay systems for key marker compounds in eye fluids. In addition, studies analyzing VEGF levels in vitreous samples did not detect differences between VEGF levels of nAMD cases and controls.[135,142]

Although the measurement of VEGF levels in vitreous or aqueous humor is expected to best reflect VEGF levels in the macula, the procedure is invasive and therefore not desirable in individuals with early or intermediate AMD. Thus, for purposes of a clinical tool for diagnosis and progression, measurement of VEGF levels in more accessible body fluids such as serum or plasma is preferable. Several studies did investigate VEGF levels in AMD patients and controls in serum or plasma, with mixed results. Four studies detected significantly upregulated levels of VEGF in serum or plasma,[6,115,205,338] but these findings are contrasted with 10 other studies that reported no association.[41,80,112,122,125,211,231,299,353,380]

VEGF signaling is mediated through a complex of receptors and coreceptors, of which the soluble form of VEGF receptor 1 has been investigated in a number of studies. As in the case of VEGF, these studies do not offer a clear direction of effect. One study investigated the levels of soluble form of VEGF receptor in vitreous and found that levels were higher in nAMD patients.[142] In contrast, 2 studies performed on serum could not corroborate these findings. One of the studies did not find any association,[256] the other even reported lower levels of soluble form of VEGF receptor in nAMD.[340]

2.1.2. *Pigment epithelium–derived factor*

Pigment epithelium–derived factor (PEDF) is produced by RPE cells and has antiangiogenic properties, opposing the effects of VEGF. It has been proposed as a target to inhibit choroidal neovascularization and its expression signature in model systems suggests that it is downregulated under hypoxic conditions.[139] Two studies on vitreous support this by demonstrating a marked reduction in PEDF levels in AMD patients versus controls.[135,142] One study analyzing aqueous humor showed the opposite result, an increase of PEDF levels in AMD patients.[334] These conflicting results are not readily explained. It is possible that in different fluids or in different parts of the eye (anterior/posterior), PEDF is regulated differently, but additional experiments are needed to determine the direction of the effect with certainty.

2.1.3. *Transforming growth factor beta*

Transforming growth factor beta (TGF-β) has been described to increase the expression of VEGF and is therefore also implicated in neovascularization.[20] In vitreous samples of nAMD patients, TGF-β was significantly elevated when compared with controls (patients with idiopathic macular holes).[20] An earlier study had already demonstrated that urinary TGF-β levels were increased in cases compared with controls, but only in early AMD was the difference significant.[121]

2.2. *Hemostatic system*

2.2.1. *Fibrinogen*

Fibrinogen is a hemorheological factor involved in endothelial functioning.[34] Abnormalities in this factor are linked to thrombogenesis and vascular disorders[166]; hence, fibrinogen has been examined for its potential involvement in AMD. Studies have yielded mixed results. A number showed that increased fibrinogen level is a significant risk factor,[65,205,223,271,321] whereas others did not find evidence for an association.[58,149,185,188,288,295,331,352,367]

2.2.2. *Plasminogen activator inhibitor 1*

Plasminogen activator inhibitor 1 is another main component of the fibrinolytic system.[29] Four studies have investigated whether a relation between plasminogen activator inhibitor 1 and AMD exists, with some support for a positive association,[367] whereas other studies did not find any association.[26,288,352]

2.2.3. *Platelet count*

Several studies have measured platelet count. Most did not find any association between platelet counts and AMD.[149,180,181,186,205]

Two larger studies did find lower platelet counts in AMD, but this minimally protective effect for platelets on the development of AMD was only significant in univariate analyses.[50,285]

2.2.4. *Von Willebrand factor*

Von Willebrand factor is a blood glycoprotein that is essential for normal hemostasis.[289] Because vascular pathology is hypothesized to be involved in the pathogenesis of AMD, Von Willebrand factor was investigated as a possible risk factor. One study showed higher levels compared with controls (but in multivariate analysis no significant correlation was found),[205] and 3 more studies found no association at all.[288,352,367]

In summary, many inconsistent results for factors involved in neovascularization have been reported, and further work is required to determine whether these could be used as AMD biomarkers. Factors involved in hemostasis described in Section 2.2 are unlikely to serve as biomarkers for AMD.

3. Oxidative stress

The human body is dependent on an aerobic environment for survival. This constant exposure to oxygen can lead to detrimental oxidative modifications of cell components and tissues. Usually, cells are equipped with sufficient antioxidative mechanisms to maintain oxidant homeostasis, but if this balance is disrupted, oxidative stress occurs.[38] Oxidative stress in cells and tissues is characterized by an excess in molecules containing free radicals such as reactive oxygen species (ROS) and reactive nitrogen species.

Polyunsaturated fatty acid (PUFA) molecules are present in lipids on the membranes of cells and are prone to oxidation due to the presence of susceptible double carbon bonds.[38,245] During the process of lipid peroxidation by ROS, the double carbon bond is oxidized, leading to the formation of unstable

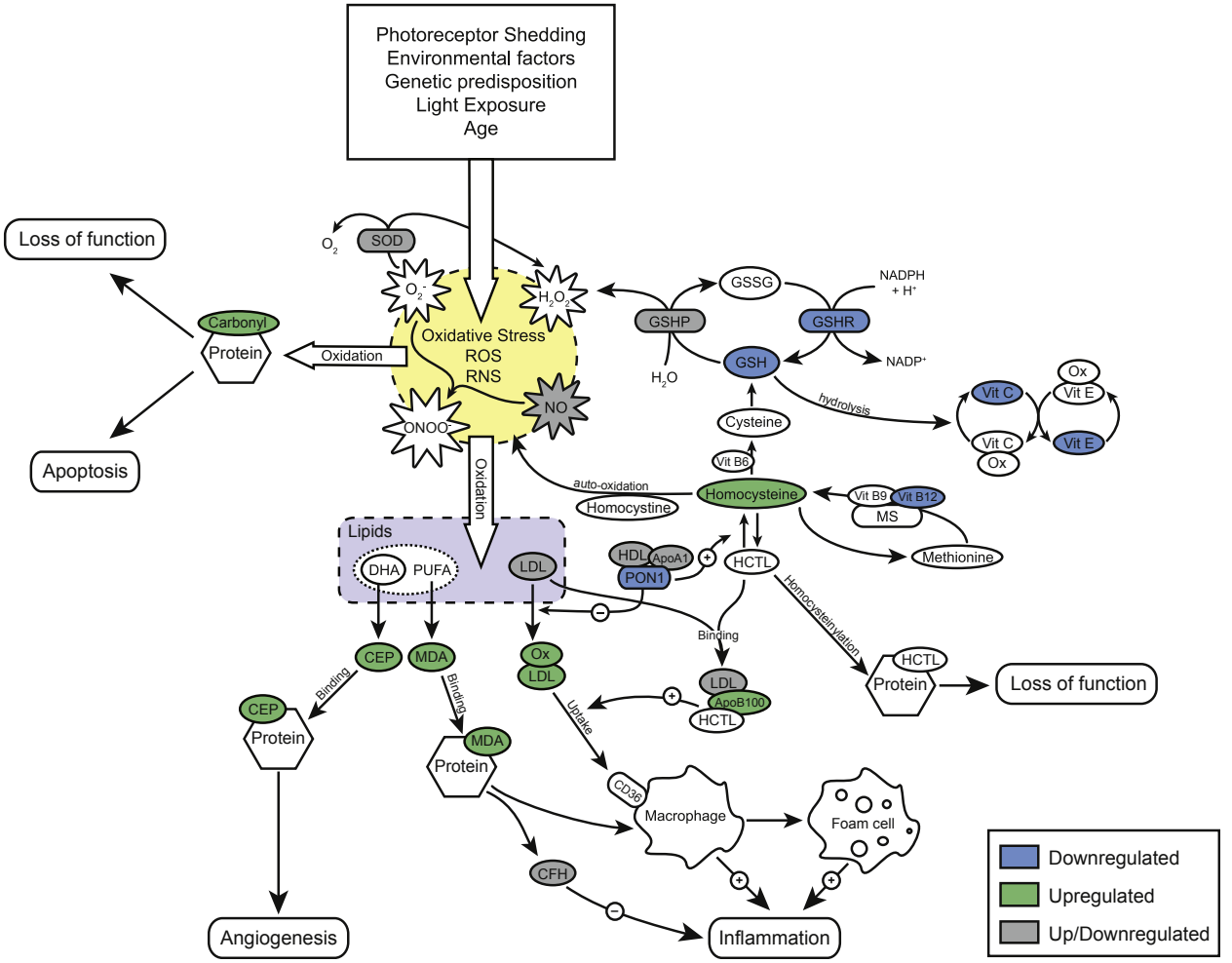

Fig. 1 – Networks of oxidative stress in age related macular degeneration (AMD). Spheres are colored to indicate levels in AMD patients compared to controls based on literature: upregulated (green), downregulated (blue), or inconsistent levels (gray). In this figure, studies reporting no association were not taken into account for the sake of readability. Apo, apolipoprotein; CEP, 2-(ω-carboxyethyl) carboxyethylpyrrole; DHA, docosahexaenoic acid; GSH, glutathione; GSHP, glutathione peroxidase; GSHR, glutathione reductase; HCTL, homocysteine thiolactone; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde; MS, methionine synthase; Ox, oxidized; PON1, paraoxonase 1; PUFA, polyunsaturated fatty acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; Vit, vitamin.

reactive carbonyl compounds (e.g., malondialdehyde [MDA]).[18,22,263,357] ROS can also oxidize proteins, resulting in 2-(ω-carboxyethyl) pyrrole (CEP) protein adducts[117] and induce formation of advanced glycosylation end products (e.g., N^{ε}-carboxymethyllysine).[146,304]

Increased oxidative stress is thought to be one of the underlying factors in the occurrence of AMD.[18,24,38,245,333,374] The eye, and especially the macula, is susceptible to oxidative stress because of its high metabolic activity and high PUFA content in the membranes of the photoreceptors.[38] High oxygen pressure from the blood in the choroid and exposure to bright light also causes increased ROS levels in the retina.[22,263,333] In addition, photoreceptors are subjected to constant shedding, and subsequent phagocytosis of the shed fragments leads to ROS generation.[38,374] Environmental factors such as smoking and alcohol consumption can also increase ROS production.[346] Therefore, factors related to oxidative stress could potentially be valuable biomarkers for the incidence and/or progression of AMD and are discussed in more detail in Sections 3.1–3.4. A schematic overview of these oxidative stress related factors is provided in Fig. 1 and a complete overview of the studies and references is provided in Supplementary Table 2.

3.1. Oxidation products

3.1.1. Malondialdehyde

MDA is one of the reactive carbonyl compounds originating from PUFA oxidation, and its presence is often used to measure lipid peroxidation levels in blood or serum samples.[18,22,335] Increased systemic levels of MDA have been consistently observed in both wet and dry AMD.[18,22,86,155,263,311,335,336,346,374,375] In addition, an allele-dependent increase of MDA levels was measured in subjects carrying the A69S variant (rs10490924) in the *ARMS2* gene that is associated with AMD. Patients heterozygous or homozygous for the risk allele showed higher MDA levels.[263]

MDA is a highly reactive molecule that forms covalent bonds with the amino acids of endogenous proteins. This MDA modification can be recognized by factors of the innate immune system such as complement factor H (FH), immunoglobulin M (IgM), and macrophages.[53,357,358] Binding of MDA by IgM or macrophages leads to a proinflammatory response by increasing the expression of the inflammation factor interleukin (IL)-8,[274,358] whereas binding to FH attenuates inflammation.[358]

3.1.2. CEP adducts and N(6)-carboxymethyllysine (CML)

Docosahexaenoic acid (DHA) accounts for about 80% of all PUFAs in the photoreceptor outer segments and is most prone to oxidation in human tissues.[96] Upon oxidative stress, DHA is oxidized forming specific CEP adducts.[117] Plasma CEP levels in AMD patients are elevated compared with controls.[118,242,351] Moreover, elevated CEP levels combined with AMD risk alleles in *ARMS2*, *HTRA1*, *CFH*, or *C3* increased the risk of AMD twofold to threefold compared with genotype alone.[118]

Furthermore, plasma of AMD patients contained more and a higher diversity of CEP autoantibodies compared with controls in 2 studies from the same group.[117,118] Another independent study found no association between CEP autoantibodies and AMD.[242]

CML is an advanced glycation end product that originates from a protein lysine modification and is a major immunological epitope recognized by the immune system.[146] Plasma CML levels were upregulated in AMD in one study,[242] but no significant difference was found in another.[304]

Both CEP adducts and CML are present on proteins. They are recognized by the immune system[146,359] and can stimulate angiogenesis in vivo.[78,248] Receptor-mediated binding of CEP adducts results in an angiogenic response of endothelial cells independent of VEGF signaling.[359] Upregulation of CML and CEP levels in AMD might be implicated in the progression toward nAMD by promoting angiogenesis, but further studies are necessary to support this hypothesis.

3.1.3. Protein carbonyl groups and total oxidation status

Levels of protein carbonyl groups are often used to assess the total protein oxidation status in subjects as they are easy to measure.[64] Protein carbonyl groups consist of an oxygen molecule bound to a carbon atom with a double bond (-RC=O) resulting from protein oxidation and are therefore indicative of oxidative stress. Elevated levels of both protein carbonyl group[336,379] and total oxidation status[336,341] were found in nAMD patients.

3.1.4. Oxidized low density lipoprotein

Low-density lipoprotein (LDL) is abundantly present in and around cells and is an easy target for oxidation by ROS. LDL cholesterol (LDL-C) has been studied extensively in the context of AMD, described in Section 5.2; however, studies on its oxidized form (oxidized low density lipoprotein [Ox-LDL]) are more limited. Higher Ox-LDL levels were found systemically in AMD patients compared with controls,[147,152,153] but a lack of association has also been reported.[184]

Increased Ox-LDL levels are known to activate various factors of the complement system such as C3b, C5b-9, and complement factor B (FB).[79] These factors are described in more detail in Section 4.1. High Ox-LDL levels as observed in AMD might initiate apoptosis of RPE cells through disruption of the mitochondrial pro- (Bax) and antiapoptotic (Bcl2) balance,[373] leading to GA. In addition, uptake of Ox-LDL molecules by macrophages contributes to the formation of foam cells, implicated in the development of atherosclerotic plaques.[270]

3.2. Nitric oxide

Nitric oxide is one of the most abundant free radicals in the human body and is able to react with other ROS resulting in cell dysfunction and apoptosis.[86] It is synthesized by endothelial cells and is an important vasoactive agent affecting blood flow and other vascular functions.[28] Involvement of nitric oxide in AMD is less clear. One study observed increased levels of nitric oxide in AMD patients,[86] another study described downregulation of nitric oxide in nAMD,[335] and a third study reported no association.[338]

3.3. Homocysteine

Homocysteine is an intermediate molecule in the conversion of the amino acid methionine to cysteine and glutathione (GSH), a process mediated by multiple enzymes.[104,294] Homocysteine

can autooxidize in plasma, leading to the formation of various reactive products such as homocysteine thiolactone, which is also accompanied by ROS generation (Fig. 1).[60]

Dysregulation of the homocysteine balance has been associated with various diseases such as vascular dysfunction, autoimmune diseases, and neurodegenerative disorders.[294] Increased systemic levels of homocysteine were observed in both neovascular and dry forms of AMD compared with controls,[18,19,60,110,113,152,153,168,213,284,300,347] and there were also higher levels in the vitreous of nAMD patients.[213] Moreover, some studies found higher homocysteine levels in nAMD compared with dry AMD[19,110]; however, other studies did not find an association between homocysteine levels and AMD.[54,132,188,247,352,367]

3.4. Antioxidants

Antioxidants enhance ROS clearance and prevent ROS formation thereby averting damage in the aging eye and other tissues.[333] Enzymes such as catalase, superoxide dismutase, and paraoxonase prevent the accumulation of oxidized lipids by converting ROS before they can react or by removing the oxidized products from the endogenous proteins.[333] Several vitamins and trace elements act as cofactors for these enzymes, or react with ROS to prevent accumulation.[357,374]

Multiple studies hypothesized that the antioxidant capacity in AMD patients might be impaired, and some showed a decreased overall antioxidant capacity in serum of patients.[58,87,269,311,336,379] In the following sections, we discuss levels of thiols (Section 3.4.1), carotenoids (Section 3.4.2), and enzymes with antioxidant activity (Section 3.4.3) in AMD patients.

3.4.1. Thiols and GSH

Thiols mediate an important part of the balance between proper oxidation versus antioxidants in tissues. Their main characteristic is a carbon-bonded sulfhydryl group (C-SH), which can form a disulfide bridge with other thiols via redox reactions (C-S-S-C). Thiols can neutralize ROS by providing an electron during the formation of the disulfide bridge.[218] Although their normal function is to prevent oxidative stress, thiols can also promote oxidative stress in the presence of metal ions such as iron.[152]

Thiol content is either measured by focusing on the individual thiols or by evaluating total thiol (tSH) content of the blood. GSH is one of the most important thiols in the body. GSH can be transformed into glutathione disulfide (GS-SG) by the enzyme glutathione peroxidase (GSHP), thereby breaking down hydrogen peroxide ($2\ GSH + H_2O_2 \rightarrow GS\text{-}SG + 2\ H_2O$).[218] Glutathione reductase (GSHR) is able to transform the formed glutathione disulfide to its monomeric form ($GS\text{-}SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$), making it available for conversion by GSHP again.[218] This circular process (Fig. 1) is of vital importance for proper ROS maintenance.

Lower levels of GSH and tSH are thought to result in more ROS formation owing to the absence of hydrogen peroxide clearance, resulting in subsequent oxidative damage.[60,333] Lower levels of total thiol content[60,152,341] and plasma GSH[60,152] were found in patients with AMD compared with control subjects, and both were negatively correlated with homocysteine levels[60]; however, multiple studies have found no association between systemic GSH levels and AMD.[35,72,273,291,375]

Plasma and serum GSHR levels were lowered in patients with AMD,[55,58,379] although 1 study did not find this association in erythrocytes.[68] Systemic GSHP levels were lowered in some studies[85,269,272,346] and higher in 1 study,[71] but in most studies, no association was found.[55,58,68,375,379]

3.4.2. Carotenoids

Carotenoids are a group of natural red and yellow hued pigments (carotenes and xanthophylls) synthesized in most plants. The antioxidant capacity of carotenoids is based on their ability to absorb and process free electrons from ROS such as singlet oxygen ($^1O_2$) and peroxyl radicals ($ROO^\bullet$). After the uptake of an electron, the carotenoid releases its energy in the form of heat and can be used again. Humans are unable to synthesize carotenoids and rely on dietary intake of vegetables.[95,325] In AMD, total serum carotenoid levels were decreased in 2 studies by the same group,[87,88] whereas 2 other studies described a lack of association.[40,313]

Two main xanthophylls are located in the macula: lutein is concentrated in the peripheral macula and zeaxanthin in the fovea. Here, they are able to attenuate blue light wavelengths, preventing the light from reaching and damaging the underlying photoreceptors.[195] In blood, lutein and zeaxanthin are transported by lipoproteins such as high-density lipopotein (HDL) and LDL. Zeaxanthin and lutein exert their antioxidant abilities by reacting with free radicals both in the macula and in blood.[195] Levels of lutein and zeaxanthin were found to be decreased in AMD patients in several studies.[70,87,384] One study described decreased levels of zeaxanthin but not lutein in AMD patients.[103] Others found no association for either lutein or zeaxanthin.[40,214,224,292,313]

β-cryptoxanthin is a carotenoid most commonly found in citrus fruits. Besides its role as an antioxidant, in vitro experiments have shown that β-cryptoxanthin also stimulates DNA repair mechanisms.[206] Levels of β-cryptoxanthin were decreased in patients with advanced AMD in some studies,[87,224,313,384] whereas others did not find a significant association with AMD.[40,70,214,292]

A decrease of α-carotene was found in patients with nAMD,[87,384] whereas higher levels of α-carotene were present in early AMD.[384] Also β-carotene levels were decreased in advanced AMD in some studies[87,224,384]; however, most studies did not find a significant association between AMD and α-carotene or β-carotene levels.[40,70,214,224,292,313,322,360] Importantly, supplementation of β-carotene has been associated with an increased risk of lung cancer in smokers and former smokers, and therefore, long-term use to inhibit AMD progression is not recommended.[5,251]

Finally, one of the most potent antioxidants present in blood is lycopene. The main dietary sources of this red pigment carotenoid are red fruits or vegetables, such as tomatoes.[102] Levels of lycopene were either decreased in AMD patients[40,313,384] or not associated with AMD.[70,87,214,224,292]

In summary, when studies reported a significant association between carotenoids and AMD, the vast majority described decreased carotenoid levels in patients. This probably reflects a difference in dietary intake of these carotenoids between AMD patients and controls. Several studies reported

that a higher intake of carotenoids is associated with a reduced risk of AMD.[332,344,365] In addition, a beneficial effect was shown for supplementation with lutein and zeaxanthin on progression to advanced AMD.[3–5]

3.4.3. *Enzymes*

3.4.3.1. *Superoxide dismutase*. Superoxide dismutase (SOD) is an important antioxidant that catalyzes the conversion of superoxide ($O_2^{\bullet-}$) into oxygen and hydrogen peroxide (H_2O_2).[333] Two families of SOD exist based on their metal ion cofactor: SOD1 (CuZnSOD), which is localized to the cytoplasm and SOD2 (MnSOD), found in mitochondria.[333] The absence of SOD1 or SOD2 has been associated with early retinal cell degeneration in mice,[129,164] suggesting an important role for SOD in the eye.

With regard to AMD, several reports show elevated systemic SOD activity in AMD patients compared with controls,[10,155,310,311] others found lowered SOD activity levels,[86,272,346,375,379] and still others measured no significant association.[55,58,68,71,269] One study showed a significant difference in SOD activity between late and early AMD, with a lower SOD activity in late AMD patients.[86]

The association of both low and high SOD serum activity levels with AMD might be explained by the damaging effects of both high and low levels of SOD. High levels of SOD lead to higher H_2O_2 production, whereas low SOD activity leads to the continuing presence of $O_2^{\bullet-}$ molecules. The detrimental effects of both low SOD and high SOD activity on ROS production suggest that imbalance of the enzyme activity leads to pathological conditions and that proper SOD balance is important to maintain homeostasis.

3.4.3.2. *Paraoxonase 1*. Paraoxonase 1 (PON1) is bound to HDL. PON1 hydrolyzes organophosphates and lipid peroxides and inhibits the oxidation of LDL.[18,153] In addition, PON1 is able to detoxify homocysteine thiolactone, one of the highly reactive metabolites of homocysteine.[151] Active PON1 interacts with oxidized proteins or lipids, leading to its own inactivation.[17] The low serum PON1 activity levels observed in AMD patients[18,22,341] could be due to inactivation of PON1 after reacting with oxidized proteins.

3.4.3.3. *Catalase*. Catalases are important in ROS clearance by converting hydrogen peroxide (H_2O_2) to oxygen and water.[47] In AMD, 3 studies reported downregulated systemic catalase activity levels,[272,346,374] whereas 3 others reported no difference in catalase activity levels between AMD patients and controls.[68,86,269]

Taken together, dysregulation of the oxidative stress pathway and the manner in which oxidative stress is managed by the body seems to play an important role in AMD. A large number of investigators have reported decreased levels of antioxidants and elevated oxidized protein or lipid levels (Fig. 1). The most promising biomarker candidates in the oxidative stress pathway are MDA and homocysteine, which were consistently reported to be increased in AMD patients. For other factors, however, the reported associations were less clear and with mixed results. This could indicate that an imbalance of the entire oxidative stress system may play a role, rather than levels of individual factors of this system specifically.

4. Immunity

The involvement of the immune system in the pathology of AMD is widely accepted, and some suggest reframing AMD as an autoimmune disease.[39] The activity of the immune system in AMD, both innate and adaptive, has been implicated at several levels. Immune cell infiltrates have been shown in the retinas of AMD patients examined postmortem,[198] with evidence of cytokine/chemokine expression at the affected site, as described in more detail in Section 4.2.

Strong evidence for the involvement of the immune system in AMD also comes from several GWAS studies (described in Section 1).[99,101] In particular, the role of the complement system is apparent. In the following sections, we discuss immunity-related compounds, including systemic markers of the complement system (Section 4.1) and elements of adaptive and innate immunity (Sections 4.2–4.4). A complete overview of the studies and references is provided in Supplementary Table 3.

4.1. *The complement system*

The complement system is an integral part of innate immunity with essential roles in protection against foreign intruders via tissue inflammation, cell opsonization, and cytolysis. It is also involved in monitoring and maintaining host tissues by clearing cellular debris, maintaining cellular integrity, tissue homeostasis, and modifying the adaptive immune responses.[105]

Ever since histopathological studies demonstrated the presence of complement components in drusen,[11,127] the involvement of the complement system in AMD has been studied extensively and genetic evidence showing strong links between components of the alternative pathway of the complement system and AMD followed.[101,193] Although the complement system acts locally, its components can also be detected systemically in serum or plasma. A number of studies have investigated the expression levels of complement regulators, complement components, and activation products in AMD patients versus controls. An overview of the alternative pathway of the complement system is provided in Fig. 2.

The central molecule of the complement system is complement component 3 (C3). Enzymatic cleavage of C3 results in the generation of its active fragments C3a (a potent proinflammatory molecule) and C3b that, via several digestion steps, leads to C3d.[220] A number of studies measured systemic C3 levels but did not find an association with AMD,[278,298,312,319] whereas higher systemic levels of its active fragments, C3a and C3d, were detected in AMD patients.[130,278,298,319] These findings suggest that the processing of C3, that is its activation, may be associated with AMD and a number of studies have investigated this. Complement activation was measured as the ratio of C3 and its degradation product C3d (C3d/C3)[280,281,319] or as a cleaved form of C3a (C3a-desArg) in blood[317] and urine.[121] Of the 5 studies that investigated complement activation in AMD, 4 found higher complement activation levels in AMD patients.[280,281,317,319] An association of C3a-desArg in urine with AMD was not established.[121] A recent study suggests that complement activation levels may

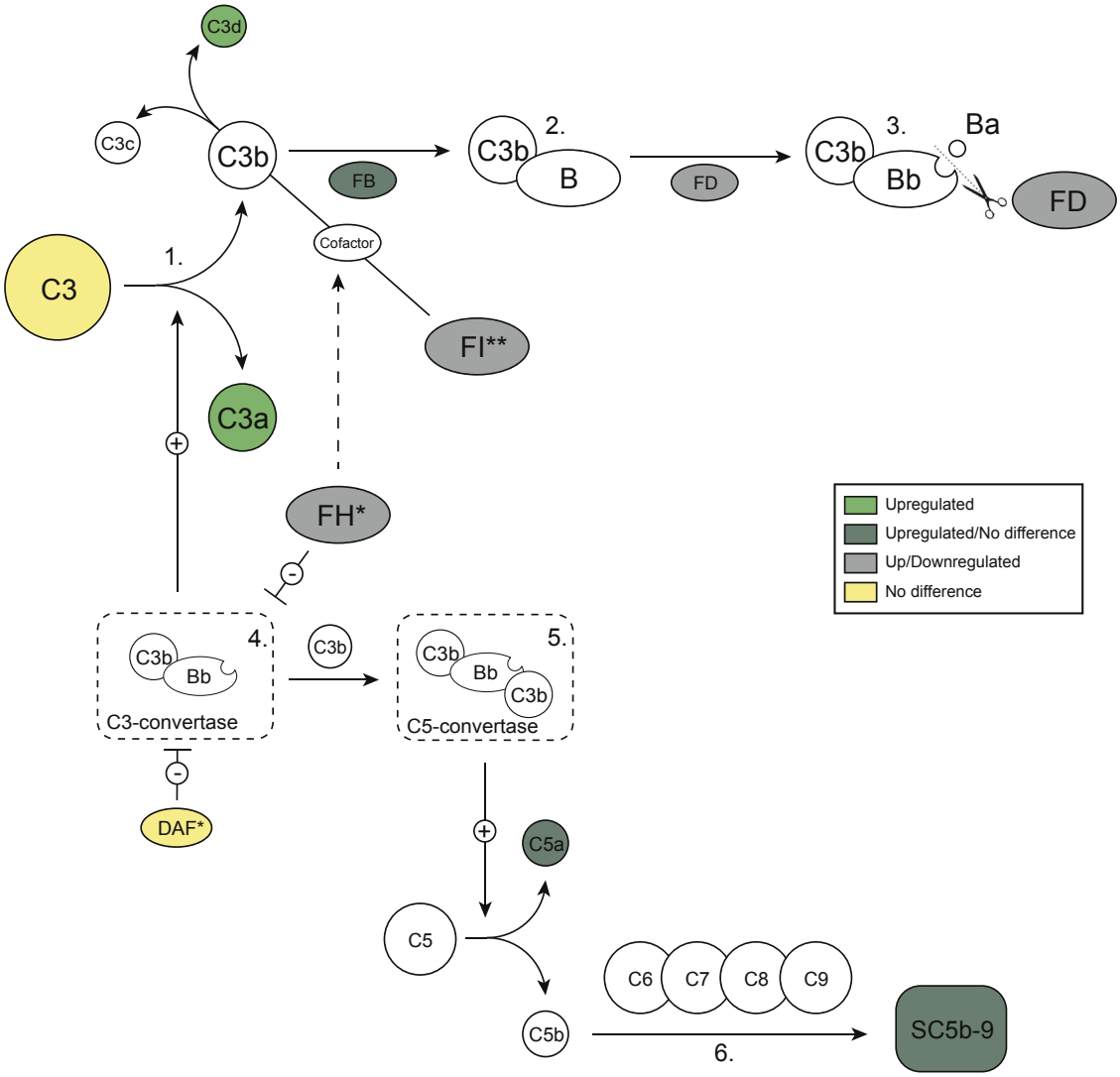

Fig. 2 – Overview of the alternative pathway of the complement system. Spheres are colored to indicate levels in AMD patients compared with controls based on literature: upregulated (green), upregulated/no difference (dark green), upregulated/downregulated (gray), and no difference (yellow). (1) Complement component 3 (C3) splits into C3a and C3b by spontaneous hydrolyzation or by the C3-convertase (C4bC2) resulting from activation of the classical or lection pathway. (2) Factor B (FB) can bind C3b to form C3bB. (3) The bound factor B is then cleaved by factor D (FD) which results in the formation of the C3-convertase: C3bBb (4). This C3-convertase can cleave C3 which leads to more C3b and in turn increased formation of the C3-convertase (known as the C3 amplification loop). The C3-convertase can also bind another C3b molecule to form C3bBb3b, which is a C5-convertase (5). This C5-convertase can convert C5 into C5a and C5b. (6) C5b then sequentially binds C6, C7, C8, and multiple C9 molecules to form the terminal complement complex (SC5b-9), also known as membrane attack complex. * The C3-convertase is inhibited by several complement regulators, among which decay accelerating factor (DAF) and factor H (FH). ** Factor I (FI) can breakdown C3b via several digestion steps to C3c and finally C3d, this protease activity, however, requires a cofactor, such as FH.

decrease at more advanced stages of the disease, but this finding needs to be confirmed in prospective AMD cohorts.[320]

Besides C3, complement component 5 (C5) is also essential in the activation cascade because it serves as the entry point for the formation of the terminal complement complex (SC5b-9).[220] The activation product of C5, C5a, is a potent anaphylatoxin. Increased levels of C5a were detected in most, but not all[130] studies examining the role of C5a in AMD.[278,298,319] These same studies also tested whether SC5b-9 is associated with AMD. Higher SC5b-9 levels were detected in AMD in 1 study,[298] but the other 2 studies found no evidence for an association.[278,319]

The activity of the complement system is tightly controlled by regulatory factors that ensure appropriate, but not excessive, generation of terminal complexes. Among others, they include complement FH (encoded by the *CFH* gene), factor I (FI, encoded by *CFI*), FB (encoded by *CFB*), factor D (FD, encoded by *CFD*), and decay accelerating factor (DAF/CD55, encoded by *CD55*).[220]

Genetic association studies showed strong evidence of an association between the *CFH* gene and AMD.[101] Systemic

levels of FH have been investigated with mixed results, however. Four studies report lower FH levels in AMD,[14,278,308,310] 1 study detected higher levels of FH in AMD,[128] and another 4 studies did not find an association with AMD.[120,298,312,319]

Similar to FH, FI also inhibits the activity of the complement system through inactivation of C3b. Genetic evidence for FI involvement in AMD has been shown previously, but no conclusive evidence links FI levels to AMD in general. One study reports increased FI levels in AMD,[312] another reports decreased levels but only in patients carrying a rare genetic variant in *CFI*,[343] and 2 did not find any association.[278,319]

The findings for FB and FD levels in AMD are also inconsistent. Three studies reported higher FB levels in AMD patients,[130,298,319] whereas 2 others did not detect an association with AMD.[278,312] Similar results were described for FD, where 3 studies reported higher FD levels,[130,298,326] 1 study reported lower levels in AMD,[312] and another found no association with AMD.[278] Finally, 2 studies that examined the role of CD55 did not find evidence for an association with AMD.[123,314]

In summary, not only genetic studies but also studies measuring complement components provide evidence that link complement activation to AMD (Table 2). Some factors, however, should be taken into account when considering the use of systemic complement activation levels as a biomarker for AMD in individual patients. Often antibody-based tests do not discriminate between the total amount of a specific complement factor and its processed activated part, as cleavage of the proform to the active mature form cannot be distinguished by the reagent. Moreover, complement activation levels are subject to high variability, and other causes of increased complement activity should be excluded because increased complement activation may reflect immune system activity that is not necessarily connected to disease progression. Linking exacerbated complement activation in an individual patient to his or her genetic blueprint is potentially more useful. For example, haplotypes and combinations of genotypes in several complement genes have been associated with increased complement activation levels.[1308,266] In addition, several investigations have now demonstrated that FI levels are lower in AMD patients carrying rare genetic variants in the *CFI* gene.[107,172,343] For FH levels, there were similar associations with genotype. Some but not all rare variants in the *CFH* gene were associated with reduced FH levels.[337,349,378] Thus, patients carrying rare variants in complement genes tend to have higher complement activation levels than AMD patients in general.[106] These insights may benefit ongoing clinical trials on the effectiveness of complement inhibitors and could prioritize patients who carry rare variants in these genes.

4.2. Cytokines

4.2.1. Interleukins

Cytokines are a large family of small proteins that play a pivotal role in cell signaling. An important group of cytokines are interleukins. Interleukins play a key signaling role in the inflammatory response. Interleukin-6 (IL-6) is a cytokine with many described functions,[215,225] and its relationship to AMD has been investigated. A number of studies reported increased levels of IL-6 in AMD patients,[7,124,191] but the majority found no association with AMD in general.[15,58,183,188,231,240,290,352,367] Notably, a number of these studies did find an association in subgroup analyses. For instance, an association with AMD was reported only in patients with high IL-6 levels[58] or the association with IL-6 was established only for GA patients.[188] In addition, only the highest tertile of IL-6 levels was associated with progression of AMD in a prospective cohort study.[303]

Other interleukins have also been studied in relation to AMD, although to a lesser extent. In most studies, these interleukins were measured in a multiplex analysis of inflammatory markers. Two studies measured multiple interleukins in serum.[231,240] In 1 study, there were higher serum levels of IL-1β, IL-4, IL-5, IL-10, and IL-13 in patients with nAMD,[240] but these factors were not associated with early, atrophic, or neovascular AMD in another study.[231] Higher serum levels of IL-1α and IL-17 in nAMD patients were only reported in the first study. In addition, no association was found for IL-2, IL-12, and IL-15.[240] Other studies also detected no association between IL-2,[188] IL-15,[89] and AMD. For IL-8, although no association was present in 2 studies,[229,235] a third larger study described higher IL-8 levels in AMD patients, in particular in dry AMD.[7] Higher IL-18 levels were reported in dry, but not nAMD, in 1 study.[143] A second study did not find different levels between different types of AMD and controls.[89]

Although most studies focused on systemic levels of interleukins, a small number performed measurements in aqueous humor[290] and vitreous.[383] Higher IL-1β levels were found in the vitreous of nAMD patients.[383] In aqueous humor, IL-1α and IL-15 were upregulated and IL-13 was downregulated, whereas for IL-2, IL-4, IL-8, IL-10, IL-12, and IL-17, no differences were detected.[290]

4.2.2. Chemokines and chemokine receptors

Chemokines (chemotactic cytokines) have the ability to direct movement of cells through receptor-mediated chemotaxis. Evidence from postmortem material and animal models have implicated infiltrating immune cells in pathological eye tissues, suggestive of the involvement of chemokines in these environments.[200,287,305,329]

Chemokine ligand 2 (CCL2; or monocyte chemoattractant protein 1) attracts C-C chemokine receptor type 2 (CCR2)-expressing monocytes into tissues and is one of the most studied chemokines in AMD. Five relatively small, case-control studies did not find an association between levels of CCL2 and AMD,[90,116,120,231,290] but several larger studies did see an association with increased levels of CCL2.[9,310,382] This effect was also reported in a cross-sectional study linking higher levels of urinary CCL2 to early AMD.[121] Overall, these findings support the notion that CCL2 is involved in AMD. Interestingly, CCR2-expressing cells can also be detected systemically, and both decreased and increased levels have been associated with AMD.[9,115] Two other studies did not find any association.[94,376]

Another receptor involved in the recruitment of monocytes, CX3C receptor 1, was measured in two AMD studies.[92,116] Only the more recent study reported CX3C receptor 1 to be upregulated in both early and neovascular AMD.[92]

Eotaxin (eosinophil chemotactic protein/CCL11) and closely related eotaxin-2 (CCL24) attract eosinophils. These are interesting molecules for AMD pathogenesis because

Table 2 – Overview of studies measuring complement components in AMD patients compared with controls

Component	Upregulation	No difference	Downregulation
C3		Scholl et al, 2008[298] Reynolds et al, 2009[278] Silva et al, 2012[312] Smailhodzic et al, 2012[319]	
C3a	Scholl et al, 2008[298] Reynolds et al, 2009[278]		
C3d	Scholl et al, 2008[298] Hecker et al, 2010[128] Smailhodzic et al, 2012[319]		
C3a des Arg	Sivaprasad et al, 2007[317]	Guymer et al, 2011[121]	
C3d/C3	Smailhodzic et al, 2012[319] Ristau et al, 2014[280] Ristau et al, 2014[281]		
C5a	Scholl et al, 2008[298] Reynolds et al, 2009[278] Smailhodzic et al, 2012[319]	Hecker et al, 2010[130]	
SC5b-9	Scholl et al, 2008[298]	Reynolds et al, 2009[278] Smailhodzic et al, 2012[319]	
FH	Hakobyan et al, 2008[128]	Scholl et al, 2008[298] Silva et al, 2012[312] Smailhodzic et al, 2012[319] Guymer et al, 2015[120]	Reynolds et al, 2009[278] Ansari et al, 2013[14] Sharma et al, 2013[308] Sharma et al, 2013[310]
FI	Silva et al, 2012[312]	Reynolds et al, 2009[278] Smailhodzic et al, 2012[319] Van de Ven et al, 2013[a,343]	
FB	Scholl et al, 2008[298] Hecker et al, 2010[130] Smailhodzic et al, 2012[319]	Reynolds et al, 2009[278] Silva et al, 2012[312]	
FD	Scholl et al, 2008[298] Hecker et al, 2010[130] Stanton et al, 2011[326]	Reynolds et al, 2009[278]	Silva and colleagues 2012[312]
DAF/CD55		Haas et al, 2011[123] Singh et al, 2012[314]	

[a] Significant downregulation of FI was described in a subgroup of patients with a rare variant in the *CFI* gene.

CCL11 and CCL24 and their receptor CCR3 are implicated in choroidal neovascularization.[91,309] CCR3 is expressed on choroidal neovascular endothelial cells and signaling through this receptor leads to endothelial proliferation, even without the involvement of eosinophils or other immune cells. Blocking CCR3 signaling in animals led to a potent inhibition of neovascularization, even stronger than blocking VEGFA signaling.[329] Levels of CCL11 were investigated in 2 studies, one reporting increased levels in AMD,[231] and the other finding no differences.[91] Supportive of the aforementioned findings, 2 studies of the same group reported CCL24 to be upregulated in AMD.[309,310] Despite these overall promising results, systemic elevations of CCR3 on immune cells have not yet been reported. The only study investigating CCR3 on granulocytes reported no association, although there was a trend toward higher expression of CCR3 in nAMD.[91] Taken together, the CCL11/CCL24-CCR3 axis is potentially involved in human AMD pathology, but it is not yet clear whether this is mostly a local signaling, mediated through CCR3 expression on endothelial cells, or whether systemic CCR3-expressing cells could also be involved.

The chemokine ligand CXCL10, also known as interferon gamma-induced protein 10, attracts a range of cell types and is an inhibitor of angiogenesis.[13] Two studies showed no association with CXCL10 in serum or plasma and AMD,[93,116] and only 1 study showed elevated serum CXCL10 levels in AMD patients.[231] Of interest is a recent publication, showing upregulation of CXCL10 in aqueous humor of AMD patients compared with controls undergoing cataract surgery,[290] suggesting that the effect of this chemokine might be local.

The receptor for CXCL10 is CXCR3 which is expressed on a variety of cell types. Only one study investigated numbers of CXCR3-expressing cells peripherally and detected reduced presence of CD8+ T-cells expressing CXCR3 in AMD,[93] but additional research is warranted before concluding whether the CXCL10-CXCR3 axis can be reliably used as a biomarker for AMD.

It has been suggested that stem cell progenitor cells are involved in the disease etiology of AMD. Chemokine ligand CXCL12, also known as stromal cell-derived factor 1, plays a role in the movement of these stem cell progenitor cells throughout the body. Four small case-control studies have investigated the plasma levels of stromal cell-derived factor 1 in AMD patients with mixed results. Two studies, by the same group, report significantly lower levels of stromal cell-derived factor 1 in patients with nAMD,[210,211] whereas another study showed the inverse effect,[299] and the fourth did not report any differences between nAMD and control individuals.[115]

4.2.3. *Other cytokines*

4.2.3.1. *Tumor necrosis factor alpha*. Tumor necrosis factor alpha, an important marker for systemic inflammation, has been investigated as such in several studies; however, no significant associations between AMD cases and controls were reported in serum or plasma.[120,124,183,188,231,240,380] Increased levels of soluble tumor necrosis factor alpha receptor 2 were reported in a case-control study in early and neovascular AMD,[89] which in a large population-based study did not reach statistical significance, but there was a trend toward upregulation in early AMD patients.[191]

4.2.3.2. *Interferon gamma*. Interferon gamma is an important cytokine in both innate and adaptive immunity as it induces cellular response to infections.[297] Three studies measured interferon gamma in AMD cases and controls, but none found an association with AMD.[89,231,240]

4.3. *Other immune factors*

4.3.1. *C-reactive protein*

C-reactive protein (CRP) is a marker of inflammation and a so-called acute phase protein because its levels change quickly upon disturbances of homeostasis. Evidence regarding the possible relation of this protein with AMD is inconclusive, with a roughly equal number of studies reporting higher CRP levels in AMD patients[7,56,57,138,174,228,282,290,301,302,304,342,347,356,376] or no clear evidence for an association.[33,58,65,120,134,141,161,185,217,285,312,315,327] Those that used a more precise measurement of CRP (high-sensitivity CRP) were also not able to provide conclusive results: 5 studies detected higher levels of high-sensitivity CRP in AMD patients,[32,124,191,230,295] compared with 5 that did not show an association with AMD.[15,183,188,352,367]

4.3.2. *(Soluble) Intercellular adhesion molecule and vascular cell adhesion molecule*

Intercellular adhesion molecule and vascular cell adhesion molecule are immunoglobulins that are usually upregulated on cell surfaces after immune signaling has taken place.[48] They form a sticky surface to which immune cells that express integrins can adhere. These molecules and their soluble counterparts are rarely investigated alone but usually as part of a panel that measures inflammatory activity. For intercellular adhesion molecule, 1 study reported higher levels to be associated with the incidence of AMD in women,[295] whereas 6 others did not find any association.[120,134,183,191,352,367] In the case of vascular cell adhesion molecule, 1 study measured higher levels in AMD patients,[191] whereas 2 studies did not find any association with AMD.[120,134] In addition, no association with AMD progression and either Intercellular adhesion molecule or vascular cell adhesion molecule was reported.[303]

4.3.3. *White blood cell count*

As mentioned previously in Section 4, a clear link with inflammation and inflammatory processes and AMD has been established, and several immune competent cells have been implicated in the disease etiology. As a result of local stress or inflammation, the body may respond by cellular proliferation of immune cells and recruitment of these cells to the affected site. From this perspective, white blood cell count is an interesting parameter to measure in AMD. A relatively large number of studies have investigated white blood cell count in AMD, and some did detect increased white blood cell numbers.[31,181,182,191,307,356] This contrasts with most studies that did not find any association.[50,113,149,157,180,181,183,185,187,205,285,315,352,367] Nevertheless, white blood cell count may still be considered as a potential biomarker for AMD if the analysis is performed in the context of a different theoretical framework. It is conceivable that it is not the total number of cells that change but rather the ratio between different cell types. Supporting this notion, a higher neutrophil/lymphocyte ratio has been associated to AMD and AMD subtypes.[148] A more in-depth analysis of the different cellular subtypes, such as the relative expression of cytokine/chemokine receptors, would offer more insights.

4.3.4. *Pentraxin-3*

Pentraxin-3 (PTX3), like CRP, belongs to the pentraxin superfamily. Upon inflammation, PTX3 is produced locally by the RPE[162] and can interact with complement component C1q and enhances activation of the classical and lectin pathways of the complement system. In addition, PTX3 attracts complement FH, thereby inhibiting the amplification loop and preventing excessive activation of the alternative pathway.[76,162] Although 1 case-control study reported higher plasma PTX3 levels in nAMD,[228] a more recent study (including also early AMD and GA patients) could not replicate these findings.[162] The latter study did however describe an increased expression of the *PTX3* gene with age- and inflammation-induced apical PTX3 secretion of the RPE.[162] Taken together, this suggests a more local expression of PTX3 in AMD; however, measurements of PTX3 locally in vitreous samples have not yet been performed and would therefore be a target of further research.

4.4. *Antibodies*

4.4.1. *Antiretinal autoantibodies*

The formation of antibodies against foreign epitopes is a key element of immunity. When endogenous epitopes become the trigger for mounting an immune response, autoimmunity ensues.[271] Antibodies against epitopes found in retinal material of AMD patients have been investigated in various studies. Several studies demonstrated upregulation of circulating antiretinal autoantibodies (ARAs) in the serum of AMD patients.[49,119,264,268] Although one study showed similar levels of ARAs in cases and controls, it did show a difference in types of antibodies specific for each disease stage.[2] In addition, higher concentrations of circulating ARAs were detected in treatment-naive nAMD patients compared with controls.[196,197] These levels also correlated to lesion size.[197] After the loading phase of anti-VEGF treatment, autoantibody levels decreased.[196,197] Moreover, correlations were reported between ARA levels and improvement of visual acuity, fluid reduction on optical coherence tomography, and decreased leakage on fluorescein angiography after 3 months.[197]

Furthermore, other studies attempted to identify specific circulating ARAs associated with AMD.[145,156,232] Surprisingly, one study showed not only upregulation of antibodies but also

downregulation of a specific ARA in AMD. Lower antibody concentrations were reported for α-crystallin, whereas α-enolase and glial fibrillary acidic protein antibodies were both significantly higher in serum of AMD patients.[156] The latter finding is supported by results from a previous study which showed different staining patterns in serum of AMD patients, with the most frequent pattern observed being almost identical to that using antiglial fibrillary acidic protein antibodies.[268] In addition, using an untargeted approach, 1 study identified 4 novel retinal antigens in serum of AMD patients: retinol binding protein 3 (Rbp3), aldolase C, pyruvate kinase isoform M2, and retinaldehyde binding protein 1.[232] Because Rbp3 and retinaldehyde binding protein 1 were previously reported in other ocular diseases, this study focused on aldolase C and pyruvate kinase isoform M2. A significant higher reactivity to aldolase C in nAMD, but not in early AMD, was reported. Because reactivity to pyruvate kinase isoform M2 was higher in both AMD groups compared with controls, this could potentially be a biomarker for the development of AMD.[232] A more recent study with a similar approach also identified ARAs with higher reactivity in AMD; heat shock 70 kDa protein 8 and 9, α-crystallin A chain, annexin A5, and protein S100-A9.[145]

4.4.2. *Other autoantibodies*

Serum autoantibodies have been extensively investigated by Morohoshi and colleagues using an antigen microarray analysis containing 85 autoantigens. Serum of AMD patients and controls showed a different IgG and IgM autoantibody profile, and multiple autoantibodies were significantly higher in AMD. In addition, they calculated IgG/IgM ratios for the antibodies and evaluated whether this ratio correlated to disease severity. Antiphosphatidylserine IgG/IgM was significantly elevated in AMD and correlated best with AMD stage. Moreover, reactivity to phosphatidylserine was highly increased in retina of AMD patients compared with controls.[232]

Other investigators focused specifically on antiphospholipid antibodies, which are reported to be found in aging people and diseases associated with aging.[257] In this study, anticardiolipin IgG levels were associated with AMD, supported by the findings of Morohoshi and colleagues which showed higher expression of anticardiolipin antibodies in nAMD compared with controls.[233,257]

As described in Section 3.1, anti-CEP antibodies have also been investigated in association with AMD.[117,118,242]

4.4.3. *Antibodies against pathogens*

Infection by pathogens leads to increased antibody titers of the foreign pathogen. Several infectious agents have been implicated in AMD, and we detail the antibodies against these pathogens in this section.

Chlamydia pneumoniae is an intracellular bacterial species that has been linked to atherosclerosis.[137] Since AMD involves inflammatory processes similar to atherosclerosis, the association of *Chlamydia pneumoniae* with AMD was explored. One small case-control study found support for this with increased antibody levels in AMD patients,[167] whereas 4 larger studies did not find evidence for a relation between anti-*Chlamydia pneumoniae* antibodies and AMD.[183,188,227,283]

The cytomegalovirus is another infectious agent that has been hypothesized to be associated with the pathogenesis of AMD, based on the relation between inflammatory processes induced by infection and the resulting vasculopathy.[227] Only 2 studies investigated this association. One found no evidence for an association,[90] whereas the other described higher levels of antibodies against cytomegalovirus in nAMD compared with controls and dry AMD.[227]

Another infectious agent possibly involved in the pathogenesis of AMD is *Helicobacter pylori*. Two studies have tested an association between antibodies against *Helicobacter pylori* and AMD but found no evidence for this, even when distinguishing between dry and neovascular AMD.[188,227]

To summarize the most important findings regarding immune-related factors, involvement of the complement system in AMD is evident and complement activation products seem to be good biomarker candidates. Increased levels of inflammatory factors, such as CCL2 or CRP, have been frequently reported and support the notion that inflammatory processes underlie AMD. Yet, these are not specifically related to AMD and may therefore not be the best biomarker for clinical implementation. The use of multiplex assays for the simultaneous detection of multiple inflammatory markers (cytokines and chemokines) holds great promise, but additional data are required to determine their usefulness as AMD biomarkers. In addition, ARAs are also associated with AMD, but at present, it is unclear whether these autoantibodies play a direct role in the etiology of the disease or rather are the result of retinal damage. Further research is therefore necessary to determine if (specific) ARAs could be used as a biomarker for AMD.

5. Lipid metabolism/homeostasis

Lipid metabolism is one of the major pathways involved in the pathogenesis of AMD as evidenced by genetic associations of lipid-linked genes *CETP*, *LIPC*, *ABCA1*, and *APOE*.[99,101] Moreover, drusen, the major hallmark of AMD, consists of at least 40% lipids.[126,350] In addition, as mentioned in Section 4.4, there are similarities in the pathogenesis of atherosclerosis and AMD.[368] Because lipids are important risk factors for atherosclerosis and CVD,[207] these might also be associated with AMD. Numerous studies have measured lipid levels in serum or plasma, and the results of these studies are summarized in Sections 5.1 to 5.4. We focus on studies that reported associations with AMD and results from large population-based studies. A complete overview of all studies and references is provided in Supplementary Table 4.

5.1. *Lipids*

Cholesterol has multiple functions. It is required for building and maintaining cell membranes, is involved in cell signaling processes, and is a precursor molecule for synthesis of steroid hormones, bile acids, and vitamin D.[131]

The population-based Cardiovascular Health Study reported lower levels of total cholesterol in AMD patients, of which the majority had early AMD.[185,217] Also in the Beaver

Dam Eye Study, lower cholesterol levels were associated with development of early AMD in women,[182] and there was a trend for lower levels of cholesterol in nAMD[186]; a more recent analysis of the Beaver Dam Eye Study data, however, did not show an association between AMD and cholesterol levels.[183] In addition, 2 case-control studies described lower levels of cholesterol in AMD patients.[36,267] In contrast, higher cholesterol was associated with AMD in 10 studies, although these were all case-control studies, and only half studied nAMD.[8,57,67,88,97,109,134,152,246,342] The vast majority of studies (Supplementary Table 4), however, did not demonstrate a difference in cholesterol levels between AMD patients and controls, including a meta-analysis of 3 large population-based studies,[190] and several large population-based studies.[33,37,42,50,61,73,141,143,157,160,161,176,177,184,199,261,304,306,321,331,345,354,367,371]

Triglycerides are molecules that have a glycerol backbone connected to 3 fatty acids of variable length. Most studies did not report differences in triglyceride levels between AMD cases and controls (Supplementary Table 4). Lower triglyceride levels were reported in early AMD,[185,371] nAMD,[219] and any AMD.[33,177,265,285,304] In contrast, 3 studies reported a higher level of triglycerides to be associated with AMD,[67,235,246] of these, 1 study included only women,[245] and 1 study found the association in women only.[235]

Phospholipids are another class of lipids and are an important component of cell membranes. In 3 studies, no association was found between phospholipids and AMD.[1,40,292]

5.2. *Lipoproteins*

Because of the insoluble nature of lipid molecules, lipoproteins are needed for transportation of lipids through the circulation. Five different lipoproteins exist, differing in their density and size: chylomicrons, very low–density lipoprotein, intermediate-density lipoprotein, LDL, and HDL.[255] Both HDL and LDL carry cholesterol between the liver and periphery.[131,208,265] The association between these 2 lipoproteins and AMD has been extensively studied.

For AMD, higher levels of LDL-C were found in several studies. Half of these studies found this association when comparing controls to nAMD,[109,152,154,279,342] others found an association in early AMD,[273] any AMD,[57,67] and in women with dry AMD.[246] Almost all other studies, including multiple large population-based studies,[33,37,50,61,184,304,331,354,371,377] did not report an association between AMD and LDL-C (Supplementary Table 4). Only the Cardiovascular Health Study associated lower LDL-C levels with early AMD patients[185] and reported a trend toward lower levels in patients with any AMD.[217] Differences in results regarding LDL-C levels can be partly due to different measurement methods across studies, as it can either be measured directly, but more often is estimated using the Friedewald equation.[98]

Since HDL cholesterol (HDL-C) is inversely associated with CVD, one may have expected to also find this inverse association with AMD. Surprisingly, lower HDL-C levels were only described in a few studies in varying AMD stages; in late AMD,[279,331] in women with dry AMD,[246] and in early AMD.[180] Increased HDL-C levels in AMD patients were present in multiple studies.[15,35,50,61,73,141,144,161,182,184,186,265,304,345,356,376] It must be noted that most of these studies only found a weak association in a subgroup of AMD patients. Most of the studies did not describe significant differences in HDL-C levels (Supplementary Table 4).

Three studies evaluated non-HDL-C, which is calculated by subtracting HDL-C from total cholesterol. Two studies, including a large meta-analysis of 3 population-based studies, reported no association with AMD,[190,265] whereas the third study found higher non-HDL-C to be associated with any AMD.[57]

Lipoprotein (a), Lp(a), is an LDL-like particle, which consists of apolipoprotein-B100 and apoliprotein-A. Its precise function is unclear, but higher levels of Lp(a) have been repeatedly associated with CVD.[82,171] Contrarily, no association of Lp(a) levels with AMD or progression of AMD has been described so far.[1,57,83,94,185,246,303]

5.3. *Apolipoproteins*

Apolipoproteins bind lipids to form lipoproteins that are responsible for lipid transport. They also function as enzyme cofactors and receptor ligands.[1] There are several classes of apolipoproteins. The overview presented in this section is restricted to apolipoprotein A1 (ApoA1), the major component of HDL-C, apolipoprotein B (ApoB), mostly found in LDL-C, and apolipoprotein E (ApoE), found in IDL-C and chylomicrons. Several investigations found an association between apolipoproteins and AMD or features of AMD.[1,73,94,246,265] The Pathologies Oculaires Liées à l'Age (POLA) study described ApoA1 to be associated with an increased risk of soft drusen[73] and also in the European Genetic Database (EUGENDA) cohort, higher levels of ApoA1 were associated with AMD, even after adjustment for genetic variants that influence lipid levels.[265] In contrast, one study reported a lower ApoA1 concentration in women with dry AMD.[246] This study also described a higher concentration of ApoB in dry AMD cases, which is in concordance with another study.[94] Higher ApoE levels were reported in advanced AMD compared with early AMD and control individuals; this difference could be due to a higher allelic burden of the *APOE* gene in these patients.[1] Other studies did not describe an association between ApoA1, ApoB, or ApoE and AMD.[57,66,83,185]

5.4. *Fatty acids*

There are different types of fatty acids. PUFAs usually derive from phospholipids or triglycerides.[245,254] The most commonly studied PUFAs in AMD are the omega-3 fatty acids DHA and eicosapentaenoic acid (EPA). Fish and other seafood are the main source of these omega-3 PUFAs.[219,221] Animal and epidemiological studies have shown a lower risk for AMD in subjects with high dietary intake of omega-3 fatty acids.[21,324] Also 2 interventional studies with omega-3 fatty acid supplementation have been performed; the Age-related Eye Disease Study 2 showed no beneficial effect for omega-3 fatty acid supplementation,[3] whereas the Nutritional AMD Treatment 2 study showed a protective effect for DHA supplementation only in patient homozygous for the major allele (T) of the Y402H variant in the *CFH* gene.[222]

Considering omega-3 fatty acids as potential biomarkers, a number of studies investigated plasma or serum levels of these factors. In the Antioxydants, Lipides Essentiels, Nutrition et maladies Oculaires (ALIENOR), a population-based study, advanced AMD cases had lower plasma levels of α-linoleic acid and DHA compared with no or early AMD. In addition, lower plasma levels of EPA were associated with GA.[221] This is in line with baseline measurements performed in the Nutritional AMD Treatment 2 study that showed that nAMD cases had lower EPA and DHA levels in red blood cell membranes and lower serum EPA.[219] On the contrary, smaller case-controls studies reported no effect or opposite effects for DHA, EPA, and α-linoleic acid.[165,252,254,292] For plasma or serum levels of docosapentaenoic acid, another omega-3 fatty acid, no significant associations were described.[165,221,292]

Omega-6 fatty acids, arachidonic acid and linoleic acid, and omega-9 fatty acid, oleic acid, have also been measured. A small case-control study found lower levels of linoleic acid and oleic acid, and higher levels of arachidonic acid in the membranes of erythrocytes of AMD patients.[254] In line with these findings, a recent study reported higher serum arachidonic acid in nAMD.[252] Two larger case-control studies, however, did not show different levels of these omega-6 and omega-9 fatty acids.[165,292]

Regarding saturated fatty acids (which are single bonded), lower levels of palmitic acid in erythrocytes of AMD patients were reported in a small, case-control study,[254] although systemic levels were not different between cases and controls.[165,254] Also for stearic acid, no association with AMD was detected.[165,254]

Evidence for the involvement of lipids in AMD comes from epidemiologic, molecular, and genetic studies, but the exact role of systemic lipid levels is not yet clear. These studies are complicated by high variability of lipid and fatty acid levels in general and are potentially further confounded by the use of medication and/or dietary intake, including supplements. Although a combination of factors could constitute a risk profile that may be linked to the development and progression of AMD, it is unlikely that these factors individually could act as proper biomarkers for the disease.

6. Extracellular matrix

Remodeling of the ECM plays a role in the pathogenesis of AMD.[158,241] Drusen development, as well as alterations of Bruch membrane[52,59] and infiltration of immune cells, relate to a balance between structural tightness or looseness of the extracellular environment. The constant remodeling of the ECM is carefully regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases.[236] Dysregulation of MMPs and/or tissue inhibitors of metalloproteinases could lead to ECM changes seen in AMD, and therefore, these are potentially useful biomarkers for AMD.

Genetic variations in several ECM-related genes are associated with AMD[99,101,275]; however, only few studies have measured plasma or serum levels of MMPs and tissue inhibitors of metalloproteinases.[45,46,120,188,381] An overview of the studies and references is provided in Supplementary Table 5. Upregulation of MMP9 in plasma was associated with AMD in 1 study[45]; however, 2 other studies could not replicate these findings.[120,381] No association was found for serum MMP1 levels[120,381] or MMP2 in serum or plasma.[45,120,381]

All 3 studies were limited because of small samples sizes and the measurement techniques used. Moreover, in these studies, both the proenzyme and active forms were measured together. Increased immunoactivity of MMPs does not necessarily mean an increase in enzymatic activity. Other measurement techniques are required to measure MMP activity more reliably, and larger future studies are needed to elucidate the potency of MMPs as biomarkers for AMD.

One of the main constituents of the ECM in Bruch membrane is elastin.[241] Elastin, in combination with other proteins of the ECM,[348] provides strong and long-lasting elasticity to the Bruch's membrane. The elastin layer degrades with age, however, and elastin metabolism may contribute to AMD where there is frequently thinning and fragmentation of the elastic layer,[52] especially in relationship to choroidal neovascularization.[31,133] There is also evidence for abnormal systemic elastin metabolism in AMD. Patients with nAMD had significantly increased susceptibility to elastolysis in the skin.[31] Patients with nAMD had significantly higher levels of serum elastin-derived peptide levels,[318] probably due to the aforementioned elevated levels of MMPs in serum.[45] Apart from elevated elastin peptide fragment levels, sera from patients with AMD contain specific autoantibodies against elastin and it has been suggested that the IgG/IgM ratio for elastin, and other, autoantibodies might allow monitoring the progression of AMD.[233] Therefore, analyzing elastin degradation products or autoantibody levels or ratios might be useful tools as biomarkers, at least for nAMD.

7. Dietary factors

Known risk factors for AMD include dietary factors, such as low intake of antioxidants. Some vitamins are antioxidants, whereas others act as cofactors for enzymes involved in ROS clearance,[333] as detailed in Section 7.1. Trace elements have also been hypothesized to be involved in the pathogenesis of AMD and are described in Section 7.2. Another marker influenced by diet is serum albumin; this is considered to be an indicator of nutritional status and inflammation and is discussed in Section 7.3. In addition, diet is also an important source for fatty acids and carotenoids both related to AMD. These are described in Sections 3.4.2 and 5.4, respectively. A complete overview of the studies and references is provided in Supplementary Table 6.

7.1. *Vitamins*

Vitamin C can act as an ROS scavenger, and it mediates reactivation of vitamin E.[333] When vitamin C hydrolyzes and reactivates vitamin E, the molecule itself is inactivated, and hydrolysis by GSH can reactivate vitamin C (Fig. 1).[258] Lowered levels of vitamin C result in less vitamin E conversion to its active form. In addition, vitamin C itself cannot fulfill its antioxidant function, and as a consequence ROS production will rise.[258] Vitamin C levels were found to be lower in AMD patients than those in controls[311] and lower in advanced versus early AMD[313]; however, most studies do not report an association between vitamin C and AMD.[30,72,87,88,360,375]

Vitamin E is anchored in the plasma membrane and prevents lipid peroxidation.[333] Lower levels of serum vitamin E in AMD patients were reported.[25,214,313,360] However, associations with vitamin E were not conclusive because no difference in vitamin E levels has been found in several studies.[31,40,72,87,88,224,292,311,322,339]

One study reported lower levels of vitamin A in patients with nAMD.[384] However, most studies did not find a significant association between vitamin A levels and AMD.[31,72,88,224,292,313,360]

B vitamins are essential molecules in homocysteine metabolism and synthesis of methionine. Both vitamin B9 (folate) and B12 (cobalamin) act as cofactors to convert homocysteine into methionine.[294] In AMD patients, lower serum levels of vitamin B12 were detected compared with controls.[113,168,284] These results were not consistently replicated, as equal levels of serum vitamin B12 in patients and controls have also been described.[132,247] Folate levels were similar between controls and AMD patients in all studies.[113,132,168,183,247,284]

Vitamin D can be produced in the dermis upon sunlight exposure or can be obtained through diet. For its activity, the molecule has to be converted into its active form in the liver and kidney before it can regulate uptake of nutrients such as iron, calcium, magnesium, and zinc.[244] There are inconsistent results for vitamin D levels in AMD patients. They have been described to be higher,[177] lower,[150,259] or not associated with the disease.[50,62,111,226,234,261,316]

7.2. *Trace elements*

Trace elements are required by the human body in very low concentrations for proper physiological functioning; however, deficiency or excess amounts may be harmful.[27]

Iron is essential for retinal functioning, as phototransduction is dependent on iron-containing enzymes. Accumulation of iron, however, can be harmful. Iron can convert hydrogen peroxide (H_2O_2) into highly reactive ROS and thereby enhance oxidative stress.[323] Cadmium can also increase ROS formation[361] and mercury can decrease oxidant defense mechanisms,[140] both leading to increased oxidative stress. In contrast, manganese, copper, and zinc contribute to antioxidant activity as they are cofactors for the antioxidant enzyme SOD.[333,362] GSHP is dependent on the presence of the essential heavy metal selenium.[17] In addition, copper and zinc are able to stabilize proteins, reducing their vulnerability to oxidation[362] but can also lead to pathological aggregation or even precipitation of proteins.[237–239] Both zinc and manganese can reduce uptake or accumulation of toxic cadmium.[293]

Several studies reported elevated cadmium levels in blood,[50,176,262,366] aqueous humor,[163] and urine of AMD patients.[366] Measurement of cadmium levels in blood might represent only recent cadmium exposure, whereas urinary cadmium reflects long-term exposure to cadmium and might therefore be a more accurate biomarker. A study comparing both blood and urinary cadmium levels did not show an association with AMD in the total study group; however, when stratified for smoking status, increased urinary cadmium levels were associated with AMD in smoking individuals, suggesting a smoke-related association of cadmium with AMD.[81] Lead levels were elevated in serum and urine of both early and advanced AMD,[50,262,366] and 1 study reported an association between lead and AMD only for women.[143] Levels of mercury were only elevated in patients with advanced AMD.[50,262]

Selenium was in general not associated with AMD.[87,88,163] One study found a borderline significant association with AMD,[339] and another measured significantly lower levels of selenium in nAMD patients.[216] Conflicting results are reported for levels of iron,[31,163,369] copper,[40,163] manganese,[163,262] and zinc.[24,88,163,262,313]

7.3. *Albumin*

Albumin is essential for maintenance of plasma colloid oncotic pressure, acts as a plasma binding protein, and also has antioxidant activity.[202] In addition, albumin is one of the most common proteins found in drusen.[63] A few studies measured serum albumin in AMD patients and controls. Two case-control studies did not show a significant association between serum albumin and AMD.[31,88] The population-based Cardiovascular Health Study and Beaver Dam Eye Study did report significantly lower serum albumin levels in early and neovascular AMD, respectively.[185,187] A more recent nested case-control study within the Beaver Dam population further analyzing these data could not confirm decreased albumin levels in AMD.[183]

Taken together, because of the highly variable diet between subjects, and varying levels of dietary factors within subjects based on fasting state, assessment of the role of these dietary factors as biomarkers in AMD remains difficult. Dietary intake and/or supplementation of antioxidants and vitamins, however, have therapeutic benefit. The Age-related Eye Disease Study trial, one of the largest investigations into vitamin supplementation in AMD, focused on daily supplementation with vitamin E, vitamin C, β-carotene, and zinc and demonstrated a lower chance of advanced AMD development in subjects taking these supplements.[4] In the Age-related Eye Disease Study 2, an improved formula was evaluated and β-carotene was replaced by lutein/zeaxanthin because of the increased risk of lung cancer in smokers.[3,5]

Regarding trace elements, toxic heavy metals (such as lead, mercury, and cadmium) are mainly associated with an increased risk of AMD, whereas essential heavy metals (e.g., zinc and manganese) seem to protect against the development of AMD. For most trace elements, there are only a limited number of studies available in the public domain to date, and further research is required to assess their potential role as a biomarker or as protective supplement.

8. Hormones

In this section, we discuss the few hormones that have been investigated in relation to AMD: leptin, melatonin, and dehydroepiandrosterone sulfate (DHEAS). A complete overview of the studies and references is provided in Supplementary Table 7.

8.1. *Leptin*

Because AMD is a multifactorial disease in which dietary factors and body mass index also play a role in the disease mechanism, it has been suggested that the principal hormone involved in

food intake behavior, leptin, may be associated with AMD. Two studies support this theory; both showed a reduction in serum leptin levels in AMD patients compared with controls.[85,306] After controlling for potential confounders, including smoking, body mass index, blood pressure, and HDL-C, the association remained significant, which suggests that mechanisms other than body fat underlie the relationship between leptin levels and AMD.[306] The third study did not observe a difference in leptin levels in patients versus control individuals.[124]

8.2. *Melatonin*

Melatonin has strong antioxidative capacities, is expressed in the retina, and expression levels decrease during aging.[173,276,277] Two studies investigated the levels of melatonin in AMD. One showed elevated blood levels of daytime melatonin in pseudophakic AMD patients.[296] The second study analyzed the major metabolite of melatonin in urine, 6-sulfatoxymelatonin, and described lower levels in AMD.[286] Comparing the 2 studies is difficult because of the differences in methodology and fluid matrix analyzed, so additional experiments linking melatonin and AMD are necessary.

8.3. *Dehydroepiandrosterone sulfate*

DHEAS is a sulfate ester of DHEA, which is an endogenous steroid hormone synthesized from cholesterol in the adrenal glands and serves as precursor molecule for sex steroids, androgen and estrogen.[212] It has been suggested that DHEAS has antioxidant effects.[212,330,342] In addition, the DHEAS level in blood decreases with age.[23,212,330] Since both oxidative stress and aging are important risk factors for AMD,[59] the question arises whether DHEAS and AMD could be correlated. Three studies investigated the association between AMD and DHEAS, all with different outcomes; higher levels of DHEAS were reported in women with early AMD,[69] another study described low DHEAS in both dry and neovascular AMD cases,[330] and a third study did not find an association between nAMD and controls.[342]

In summary, only a limited amount of studies assessing hormones in AMD have been performed with inconclusive results and do not seem to be reliable biomarkers for AMD at this point in time.

9. Factors related to comorbidities

AMD has been suggested to share risk factors or coexist with other diseases, such as kidney disease, diabetes mellitus, and Alzheimer's disease. Factors related to these comorbidities are discussed in Sections 9.1–9.3, respectively. Although AMD has not been associated with liver disease before, some studies investigated factors related to liver function and these are described in Section 9.4. A complete overview of the studies and references is provided in Supplementary Table 8.

9.1. *Kidney disease*

Several studies have suggested overlapping risk factors between AMD and kidney diseases.[77,189,203,356] A number of large, often population-based, studies have not only investigated kidney function, such as glomerular filtration rate, but also markers that can be measured in serum/plasma like creatinine and cystatin-C. In the Beaver Dam Eye Study, serum cystatin-C was associated to the incidence of early AMD and nAMD.[189] In the Multi-Ethnic Study of Atherosclerosis, this association was only found when the highest deciles of cystatin-C were compared with other deciles with prevalence of early AMD.[51] In the Hatoyama study, no association between cystatin-C and AMD was found.[15]

Several large studies investigated creatinine in patients, but no clear association between serum creatinine and AMD was found. Two reports from the Korean National Health and Nutrition Examination Survey describe a significant difference between AMD patients and controls, but after adjustment for other variables, no significant association was found.[50,261] The remainder of the studies, including large population-based studies such as the Multi-Ethnic Study of Atherosclerosis and the Singapore Malay Eye Study, did not find any association between serum creatinine and AMD.[31,33,37,150,152,153,247]

Another indicator of renal health is blood urea nitrogen, but also for this factor, no link was established with AMD.[31,50,189,261]

9.2. *Diabetes mellitus*

Although some cardiovascular risk factors, such as smoking, have been consistently related to AMD, there are conflicting results for an association between diabetes mellitus and AMD.[43] Several studies, mostly population-based, measured glycated hemoglobin and glucose as indicators for the presence of diabetes mellitus. Only one study found lower levels of glucose in advanced AMD,[199] but none of the other studies described an association of either markers with AMD.[31,33,37,73,88,143,152,153,160,176,321,371,377] Several studies, all reports from the Korean National Health and Nutrition Examination Survey, reported lower glycated hemoglobin levels in AMD[50,143,176,177,199]; however, studies from other cohorts detected no difference.[33,37,160,342,380]

9.3. *Alzheimer's disease*

Similar to AMD, the prevalence of Alzheimer's disease increases with age. This neurological disorder is characterized by amyloid plaques in the brain, with the main component being amyloid beta (Aβ).[16] In AMD, 2 studies identified Aβ as a component of drusen.[12,75] In addition, Aβ might trigger activation of the complement cascade in AMD.[159] Several isoforms of Aβ with different amino acid lengths exist; in this section, we discuss the most common isoforms: Aβ1-40 and Aβ1-42.

A small, case-control study did not show different levels of Aβ1-42 between controls and either dry or neovascular AMD[247]; however, 2 more recent case-control studies showed significantly higher Aβ1-42 peptide levels in AMD patients.[120,124] Also after correction for age, Aβ1-42 was significantly associated with AMD, and there was a trend toward increasing levels of Aβ with increasing disease severity.[120] An association of AMD with Aβ1-40 in these studies was less clear. A significant upregulation was described in one study in

nAMD only,[120] whereas the other study did not report a difference between nAMD patients and controls.[124]

9.4. *Liver function*

So far, to our knowledge, no study has focused specifically on liver function and AMD. In a few studies, indicators of liver function have been reported as part of a routine blood examination with no associations between lactate dehydrogenase, aspartate transaminase, or alanine transaminase and AMD.[31,50,285]

For hepatitis B surface antigen on the other hand, an association was described in several Korean studies, a country where hepatitis B is still endemic.[50,261,285] In these studies, hepatitis B surface antigen carrier status was positively associated with AMD. Hepatitis B surface antigen has been detected in subretinal fluid, and it is hypothesized these individuals are therefore at increased risk for uveoretinal pathology, such as AMD.[261,285]

In conclusion, despite coexistence and overlapping risk factors with AMD, biomarkers for kidney disease, diabetes mellitus, and liver disease discussed here do not seem good biomarker candidates for AMD. As an exception, Aβ could potentially be a marker of disease progression; however, larger prospective studies are required to confirm these findings. In addition, also in terms of a potential new drug target, further evaluation of this biomarker in AMD seems worthwhile, as promising anti-Aβ therapies are being developed for Alzheimer's disease.[16]

10. Hypothesis-free techniques

In the past decade, many advanced high-throughput omic technologies have been developed. These technologies enable us to analyze large numbers of markers at the same time in an untargeted and unbiased manner. Here, we discuss several omic technologies in association with AMD (Fig. 3): proteomics (Section 10.1), metabolomics (Section 10.2), and epigenomics (Section 10.3). Expression of circulating microRNAs can also be measured using high-throughput techniques; these are described in Section 10.4.

10.1. *Proteomics*

The field of proteomic research uses mass spectrometry, or variations to this technique, to determine the nature of peptides or proteins in various tissues or other biological samples. The advantage of proteomic research is that it delivers results that are unbiased by preconceived notions or hypotheses. Within the field of AMD, proteomics has been used in a number of investigations, and several have been successful in showing particular proteomic signatures in plasma, vitreous, and aqueous humor from AMD patients when compared with controls.

A small study by Kim and colleagues identified 154 proteins in aqueous humor of 9 nAMD patients and 8 cataract controls.[178] In this study, 7 potential biomarker candidates were selected for further analysis: ceruloplasmin, PEDF, plasma protease C1 inhibitor, TGF-β1, clusterin, cathepsin D, and cystatin D. The relative abundances of TGF-β1, plasma protease C1 inhibitor, ceruloplasmin, and PEDF were shown to be significantly higher in AMD samples compared with controls. Another small study, collecting and profiling aqueous humor of 6 nAMD patients and 6 cataract controls, found 68 proteins to be differentially expressed.[372] Only 9 proteins were identified in both studies, among which were some that were related to AMD previously (CCL24 and complement FI), lipocalin-1 and several members of the crystallin family. These crystallins, known for their chaperone function, may also be involved in protein-protein interaction, prevention of apoptosis, and inhibition of inflammation among others.[170] Lipocalin-1 concentrations were quantified using enzyme-linked immunosorbent assay, and levels were significantly elevated in the aqueous humor of nAMD patients.

A third small study performed a focused proteomic analysis on protein members of the ubiquitin pathway.[201] Difference in expression of 6 proteins in aqueous humor of 2 AMD patients compared with 2 controls was reported. This included the 26S proteasome non-ATPase regulatory subunit 1 (Rpn2), a protein that is also present in plasma. Rpn2 was therefore selected as potential AMD biomarker and liquid chromatography-multiple reaction monitoring mass spectrometry of another 15 aqueous humor samples showed a relative increase of Rpn2 in nAMD patients.

Kang and colleagues analyzed aqueous humor samples of 26 treatment naive patients with nAMD and 18 controls.[169] By comparing expression profiles in exosomes of aqueous humor and cultured RPE cells, 6 candidate proteins were selected for verification in an independent sample set by liquid chromatography-multiple reaction monitoring mass spectrometry: actin, myosin-9, heat shock protein 70, cathepsin D, cytokeratin 8, and cytokeratin 14. Of these, cytokeratin 8 showed the highest area under the curve value (0.929), suggesting that it is a strong predictor for AMD. Although cytokeratins were not previously reported in other proteomic analyses in AMD and might be valuable markers to further investigate, it is disputable whether they could qualify as manageable biomarkers. Cytokeratins are abundant contaminants in laboratories,[209] so careful replication of these findings in other laboratories is warranted.

One other study investigated in a targeted manner the involvement of Wnt modulators in aqueous humor and found that WNT inhibitory factor 1 (WIF-1) and Dickkopf-related protein 3 (DKK-3) were upregulated in nAMD.[260]

In a study of 73 nAMD patients and 15 controls, a large set of proteins were detected in vitreous humor, of which 19 were upregulated in nAMD patients.[194] Bioinformatic analyses suggested enrichment of the complement and coagulation cascades, as well as markers involved in arachidonic acid metabolism. Of the 19 proteins, 5 were randomly selected for Western blot validation; alpha-1-antitrypsin reached statistical significance, whereas ApoA1 and transthyretin showed a nonsignificant increase in AMD. These findings need validation in a larger sample set.

Nobl and colleagues investigated vitreous samples of 108 nAMD patients and 24 controls, distributed over a discovery and validation set, and discovered 101 different proteins.[243] Using a closed testing procedure, they focused on 4 differentially expressed proteins as candidate AMD biomarkers:

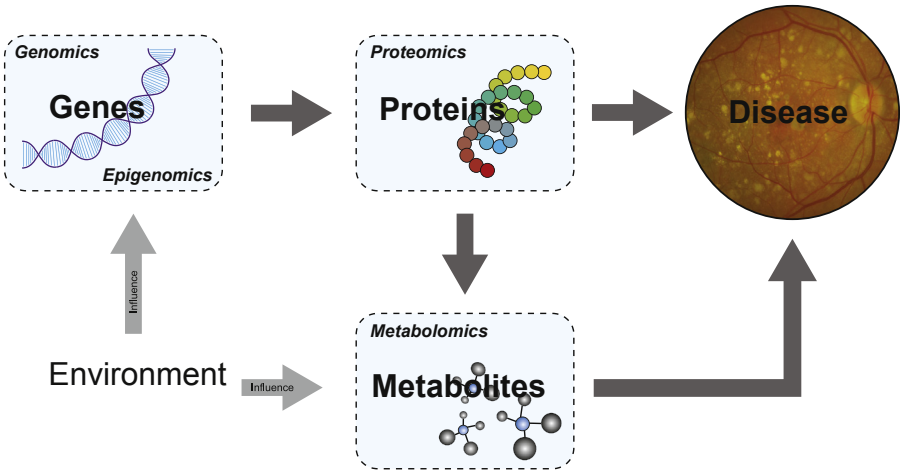

Fig. 3 – Omics in age-related macular degeneration.

clusterin, opticin, PEDF, and PH2D, which were increased in nAMD compared with controls, except for opticin, which was reduced. Upregulation of PEDF and PH2D in nAMD was described previously.[178,194] Clusterin and PEDF remained significantly increased in nAMD after validation and correction for multiple testing in an independent sample set using enzyme-linked immunosorbent assay.

There have been limited plasma proteomic studies. Xu and colleagues found 28 clinically relevant proteins to be altered in AMD patients (N = 24) compared with healthy volunteers (N = 6),[370] but further investigation of these plasma proteins is necessary to validate these findings. In addition, 2 studies using proteomic profiling of the same data set identified 3 potential AMD biomarkers: vinculin, phospolipid transfer protein, and mannan-binding lectin protease-1.[175,179] In general, proteomics of plasma or serum is a great analytical challenge due to the dominant fraction of highly abundant proteins, which have effectively prevented the discovery of novel proteomic biomarkers in these fluids in the past. Therefore, improved technologies are needed. Fortunately, some progress has been made using quantitative shot-gun proteomics, recently.[108]

10.2. *Metabolomics*

Metabolomic studies use mass spectrometric technologies or nuclear magnetic resonance spectroscopy to measure derivatives of metabolism. The technique offers a snapshot of the physiological state of an organism at the level of body fluids (urine, tears, serum, and plasma), cells or even tissues. Metabolomic analysis of AMD has great potential to uncover novel pathways in the disease that are reflective of the interaction between the genetic blueprint of individual and environmental factors that influence the metabolites (e.g., diet and smoking). To date, only one metabolome-wide study was conducted in plasma samples of 26 nAMD patients and 19 controls. Pathway analysis pointed toward involvement of tyrosine metabolism, urea metabolism, and vitamin-D–related metabolism.[253]

10.3. *Epigenomics*

Although it is clear that both genetic components as well as environmental elements contribute to the risk of developing AMD, it is less clear how these 2 systems interact. This interaction is the domain of epigenetics, induced changes in the expression levels of genes controlled by outside influences. Epigenetics is a broad term, encompassing many possible regulatory mechanisms of gene expression. One type of epigenetic mark that has been explored in a number of studies is the difference in DNA methylation patterns between cases and controls.

Epigenetic changes can be observed in peripheral blood leukocytes, which are relatively easy to obtain. One study showed a decrease in methylation near the IL17RC promotor region, suggesting that this could serve as a potential biomarker for AMD.[355] However, the finding could not be validated by an independent study with a sufficiently powered study design.[249]

Based on these results, and also because epigenetic mechanisms are likely to be tissue specific, the relationship between DNA methylation patterns in peripheral blood and retinal tissue was investigated in a recent study.[250] Although no epigenome-wide association peak was observed, the study did report consistent methylation changes across multiple samples near the *ARMS2* locus and near the protease serine 50 (*PRSS50*) gene.

Despite a limited sample size, the results provided some evidence that methylation patterns in blood leukocytes could serve as proxies for retinal changes, implying that such studies could deliver additional biomarkers for AMD.[250]

10.4. *Circulating microRNAs*

A microRNA (miRNA) is a small noncoding RNA molecule that regulates gene expression after transcription, thereby influencing biological processes. These miRNAs are present in circulation and could potentially serve as biomarkers.[229] Because we focus on compounds found in body fluids, only

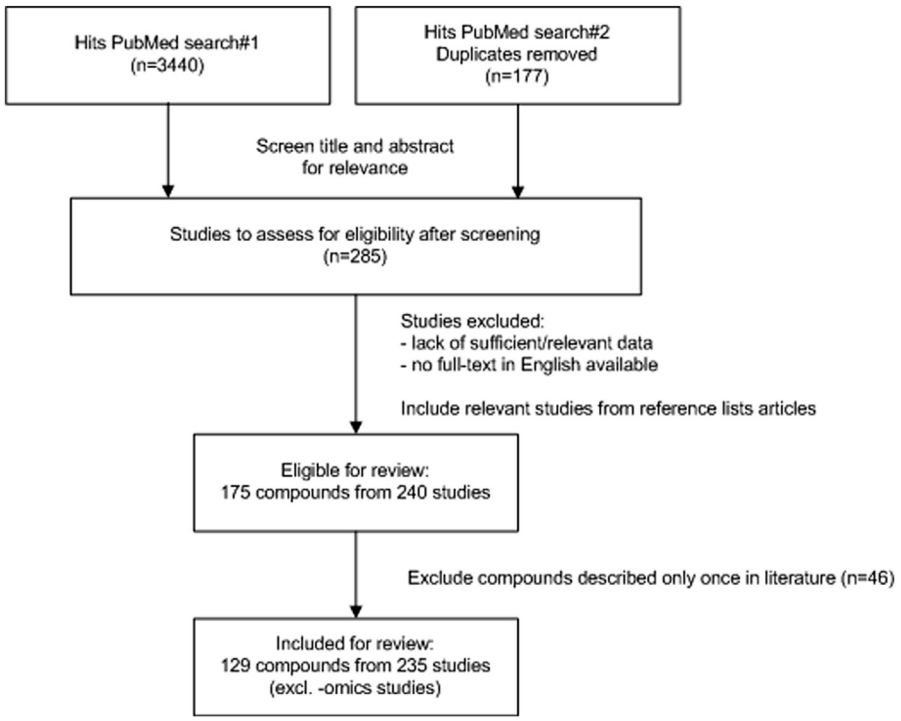

Fig. 4 – Flow diagram of literature search. The screening and selection process of studies included for this review is depicted in the flow diagram.

the studies that investigate circulating miRNAs (cmiRNAs) in serum or plasma are described here.

In a small study by Ertekin and colleagues,[84] plasma samples of 33 nAMD patients and 31 controls were analyzed for the expression of 384 miRNAs. They found 16 miRNAs to be differentially expressed between the 2 groups and additionally discovered 10 miRNAs to be only expressed in nAMD patients.

Grassmann and colleagues identified 203 cmiRNAs in serum, of which 3 (hsa-mir-301-3p, hsa-mir-361-5p, and hsa-mir-424-5p) were significantly altered in nAMD patients (N = 129) compared with control individuals (N = 147).[114] No significant association was found in GA patients (N = 59), suggesting different mechanisms for advanced AMD subtypes. Pathway analysis of the genes that are likely regulated by the altered cmiRNAs implicated the mTOR and TGF-β pathways in nAMD and knockdown of these cmiRNAs in vitro resulted in increased angiogenesis but only significantly for hsa-mir-361-5p.

Szemraj and colleagues also reported significant differences in cmiRNA profiles between dry and neovascular AMD patients.[328] In this study, serum expression levels of 377 miRNA genes in 300 AMD patients (150 nAMD/150 dry AMD patients) and 200 control individuals were analyzed. This study identified 31 differentially expressed miRNAs between patients and controls, including 2 of the 3 previously associated[114] cmiRNAs (hsa-mir-301-5p and hsa-mir-424-5p). Of the differentially expressed miRNAs in this study, 5 were significantly different between patients with dry and neovascular AMD. In addition, the correlation between these miRNAs and expression of VEGF and VEGFR2 was assessed, and it was suggested that miRNA Let-7 is implicated in the neoangiogenesis in nAMD.

So far, limited studies on miRNA profiling in AMD have been performed and results need to be replicated in larger studies; however, these initial findings emphasize the potential of cmiRNAs as biomarkers in AMD.

In general, studies using hypothesis-free techniques demonstrate proof of concept that omic analyses are able to identify novel biomarkers for AMD; however, more are needed to validate results and to confirm the clinical utility of these biomarkers.

11. Conclusion and future directions

In summary, numerous compounds have been analyzed in relation to AMD. However, only a few of these have potential as AMD biomarkers. The most promising biomarker candidates belong to the oxidative stress pathway, the complement system, and to a lesser extent, lipid metabolism. Finally, the use of hypothesis-free techniques in biomarker detection holds great promise. For summarized findings regarding factors belonging to the other biological pathways described in this review, we refer to the closing paragraphs of the respective chapters. As of yet, none of the biomarkers that we have reviewed here are used clinically.

Many studies reported decreased antioxidant levels and elevated levels of oxidized proteins or lipids indicating oxidative stress in AMD. MDA is often used as a marker for lipid peroxidation, and increased levels of MDA have been very consistently observed in both wet and dry AMD (11 of 11 studies, Section 3.1). In addition, most studies reported higher levels of homocysteine, an intermediate in the oxidative stress pathway, in AMD (12 of 18 studies, Section 3.3). Besides

dysregulation of the oxidative stress pathway, many studies indicate the involvement of the complement system in AMD. Products of complement activation and levels of complement activation—described by the ratio of C3 and its degradation product C3d (C3d/C3)—were repeatedly associated with AMD (Section 4.1). In addition, there is clear involvement of lipids in AMD from genetic and molecular studies; however, the role of systemic lipids in AMD is not fully elucidated, and therefore, they are not yet applicable as robust biomarkers for the disease.

In general, many inconsistencies exist between studies evaluating biomarkers and their association with AMD. The contradicting results are difficult to interpret due to a variety of differences between studies, including methodological differences (fasting vs nonfasting blood), different populations (Caucasian/Asian/Mediterranean) with different dietary habits, different study designs, different analytical methods, and correction factors, but also types of AMD included in the studies. It must be noted that compiling and comparison of data deriving from different sources represent a major limitation. Therefore, large well-conducted prospective studies are needed to further clarify these results.

Although AMD represents a phenotype restricted to the eye, many studies have investigated systemic markers in relation to AMD; however, because of the presence of the blood-retinal barrier, biomarkers might be only locally dysregulated inside the eye without a measurable systemic effect. In addition, some compounds are differently expressed between tissues, leading to different results when analyzing different matrices. One might therefore argue to measure markers only locally; however, because of the invasive character and accompanying ethical issues, systemic markers are preferred for implementation as clinical biomarkers.

Until now, most studies have targeted specific single biomarkers in a candidate-driven approach. Omic studies with an unbiased view are heavily outnumbered. Future biomarker research should therefore combine hypothesis-free as well as candidate-driven approaches. Quantitative analytical approaches applied in an untargeted and targeted fashion, such as metabolomic or proteomic studies, are necessary to identify novel biomarker candidates. Once validated as robust and reliable markers, they can offer more insights into the etiology and pathogenesis of AMD and support prediction, diagnosis, stratification, monitoring of treatment, and drug development for AMD.

Other biomarker types in AMD such as genetic factors, imaging biomarkers, or visual function measurements are currently of key importance for proper clinical diagnosis, stratification, and treatment of AMD. In the future, these established clinical examinations and diagnostic tests may well be applied in combination with molecular biomarkers, an area which is still in a nascent stage.

12. Methods of literature search

A review of literature was performed through a thorough PubMed search in November 2015. We used the following keywords and their synonyms in various combinations: age-related macular degeneration, serum, plasma, blood, urine, tear, aqueous, and vitreous. No limitations were set for the time range covered by our search, and therefore, all articles published until our search were included.

All abstracts were screened for relevance and full texts of the selected articles were studied. We included only articles written in English. Articles cited in the reference lists of articles obtained through this search were also included whenever relevant. Animal, ex vivo, and in vitro studies were excluded. To include the most recent developments before submission, the search was repeated in June 2016. An overview of our selection process is detailed in Fig. 4.

After the final article selection, all described compounds in these studies were grouped based on their common biological function or pathway, and results were discussed accordingly. Of note, compounds that were only described once in literature were not mentioned in this review to reduce the effect of selective reporting.

13. Disclosures

The authors do not report any proprietary or commercial interest in the subject matter of this paper.

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Supplementary data

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